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Unlinked genetic loci control the reduced transcription of aminopeptidase N 1 and 3 in the European corn borer and determine tolerance to *Bacillus thuringiensis* Cry1Ab toxin☆

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ABSTRACT

Transgenic expression of *Bacillus thuringiensis* (Bt) crystalline (Cry) toxins by crop plants result in reduced insect feeding damage, but sustainability is threatened by the development of resistance traits in target insect populations. We investigated Bt toxin resistance trait in a laboratory colony of the European corn borer, *Ostrinia nubilalis*, selected for increased survival when exposed to Cry1Ab and correlated survival on Cry1Ab toxin with a constitutive ~146.2 ± 17.3-fold reduction in midgut aminopeptidase N1 (*apn*1) transcript levels. A 7.1 ± 1.9-fold reduction *apn*3 transcript level was also correlated with Cry1Ab resistance. Quantitative trait locus (QTL) mapping identified a single major genome region controlling Cry1Ab resistance on linkage group 24 (LG24), and a minor QTL on LG27. Both QTL were independent of *apn*1 and *apn*3 loci on LG02. Positional mapping identified genetic markers that may assist in the identification of causal gene(s) within QTL intervals. This study indicates that genetic factor(s) may act in *trans* to reduce both *apn*1 and *apn*3 expression in Cry1Ab resistant *O. nubilalis* larvae, and suggest that gene regulatory pathways can influence Bt resistance traits. These findings show that gene interactions (epistasis) may influence Bt resistance in target insect populations.

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1. Introduction

The transgenic expression of crystalline (cry) protein toxins derived from the soil bacterium *Bacillus thuringiensis* (Bt) by crop plants are a success story for biotechnology. Genetically engineered (GE) plants that express Bt toxins can dramatically reduce feeding damage caused by target insects, and have likely been a cause of decreased pest insect population sizes and levels of crop damage (Hutchison et al., 2010). These benefits have contributed to a high rate of GE crop adoption by producers, such that by 2010 GE corn comprised ~63% of the 35.6 million hectares planted in the United States (USDA-NASS 2010). Continual exposure of insect populations to high levels of a insecticide can lead to the selection of tolerant or resistant phenotypes (Labbé et al., 2007), and the potential for resistance development has been a concern since the commercialization of Bt crops in the late 1990s. Resistance was first documented in the lepidopteran insect species *Plodia interpunctella* (McGaughey, 1985), *Plutella xylostela* (Tabashnik et al., 1990), and *Tricoplusia ni* (Janmaat and Myers, 2003) in response to selection caused by applications of foliar Bt sprays. Initial description of resistance traits and subsequent modeling of potentials for accumulation of resistance phenotypes in pest insect populations were based on what is referred to as "Mode 1" Bt resistance. "Mode 1" resistance traits in Lepidoptera are defined a being recessive, showing >500-fold increases in resistance compared to wildtype individuals, having negligible cross-resistance to Cry1C and showing reduced binding of Cry1A toxins to midgut epithelium due to action of a single gene (Tabashnik et al., 1998).

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Insect resistance management programs aimed to delay the accumulation of Bt resistance traits in pest insect populations have used the high-dose/refuge (HDR) strategy (Alstad and Andow, 1995; US EPA, 2001). A "high dose" of Bt toxin is hypothesized to cause mortality among 100% of homozygous susceptible and heterozygous larvae that feed on the GE crop plant. In conjunction with a "high dose", the HDR strategy relies on planting of non-Bt (conventional) crop plants that functions as a repository where an excess of susceptible phenotypes will develop to adulthood. When refuges are planted in proximity to Bt corn, a large number of susceptible individuals are likely to mate at random with rare homozygous resistant adults that might emerge from Bt fields. Mode 1 resistance forms the premise behind the HDR strategy that is used for insect resistance management of Bt resistance in populations of pest insects (US EPA, 2001). Despite of required implementation of IRM programs for Bt product registrations, field-evolved resistance to transgenic Bt corn expressing the Cry1Ab toxin has been shown in populations of *Busseola fusca* (van Rensburg, 2007) as well as Bt hybrids that express Cry1F toxin by *Spodoptera frugiperda* (Matten et al., 2008). Additionally, cotton bollworm populations show resistance to Cry1Ac-producing cotton in India (Kranthi, 2005), China (Li et al., 2007), and the southern United States (Tabashnik et al., 2008). Although not detected in field settings, a field-derived laboratory colony of *Ostrinia nubilalis* is known to survive and develop on corn hybrids that express Cry1F toxin (Pereira et al., 2008).

The molecular genetic basis of "Mode 1" Bt resistance has been shown to result from structural mutations in insect receptor proteins (Gahan et al., 2001, 2010) or a reduced expression of a single toxin receptor gene (Zhang et al., 2009; Herrero et al., 2005; Tiewsiri and Wang, 2011). For example, aminopeptidase N (*apn*) genes expressed in the midgut encode epithelial membrane-bound enzymes that are also bound by Cry1Aa, Cry1Ab, and Cry1Ac (Chang et al., 2008; Zhang et al., 2009), as well as Cry1F (Hua et al., 2001) and Cry1C toxins (Herrero et al., 2005). Reduced expression of *apn*1 transcripts was shown to eliminate Cry1C binding to *Spodoptera exigua* brush border membrane vesicles and was correlated with larval resistance to Cry1C (Herrero et al., 2005). Additional single gene Bt resistance traits were shown to result from mutations in the midgut epithelial membrane-anchored proteins ABC transporter (*abcc*), alkaline phosphatase (*alp*), or cadherin (*cad*) (Gahan et al., 2001, 2010; Jurat-Fuentes et al., 2002; Baxter et al., 2011) of larval Lepidoptera. Cry1Ac resistance in the YHD2 strain of *Heliothis virescens* was shown to involve mutations in both *cad* and *abcc*2 genes (Gahan et al., 2001, 2010). Despite this knowledge, the genetic basis of Bt resistance remains difficult to discern in many cases, where Baxter et al. (2008) and Coates et al. (2011a,b) also reported that Cry1 toxin resistance traits are conferred by loci that do not include genes that are known to encode proteins which are bound by Bt toxins.

Complexities of the molecular mechanisms involved in the expression of "Mode 1" resistance in Lepidoptera was shown by characterization of genetic factors that form part of a *trans*-regulatory pathway that alters the expression of the *apn*1 Bt binding receptor in Cry1Ac resistant *T. ni* (Tiewsiri and Wang, 2011). In the current study we also show that down-regulation of *apn*1 and *apn*3 transcripts in midgut are linked to increased survival of larvae from a laboratory colony of *O. nubilalis* when exposed to Cry1Ab toxin on artificial diet. Furthermore, the genetic control of Cry1Ab resistance by two QTL are independent of aminopeptidase N loci identifies a complex and polygenic mechanism of *trans*-regulatory control of Bt resistance in Lepidoptera.

2. Materials and methods

2.1. Dose-response assays and dominance of Cry1Ab resistance

A laboratory colony of *O. nubilalis* with a ≥2500-fold increase in Cry1Ab-toxin tolerance compared to susceptible controls (colony Cry1Ab^R) was previously selected by Coates et al. (2007), but toxin dose–response assay data were not reported. Cry1Ab toxin overlay bioassay treatments of 0.0, 1.0, 2.5, 5.0, 7.5, 10.0, 25.0, 50.0, 75.0, and 100.0 ng Cry1Ab toxin × cm^{-2} were prepared as described by Marçon et al. (1999). Three replicates of 16 *O. nubilalis* neonates from a Cry1Ab susceptible colony (Cry1Ab^S) were exposed to Cry1Ab toxin on overlay diet for 7 d. Similarly, three replicates of 16 neonates from Cry1Ab^R reared on 0.0, 10.0, 100.0, 500.0, 1000.0, 5000.0, 7500.0, 10000.0 and 12500.0 ng Cry1Ab toxin × cm^{-2} for 7 d. Survival was scored as larvae that molted to ≥2nd instar, and lethal concentration required to cause 50 (LC50) and 99% (LC99) mortality were calculated according to Marçon et al. (1999).

Dominance of the Cry1Ab resistance trait was estimated from cross started between ~50 Cry1Ab^R and ~50 Cry1Ab^S adults. Subsequent reciprocal backcrosses were preformed *en masse* between ~25 ♀ F_1s (rS) and ~25 ♂ from the Cry1Ab^R (rr) or Cry1Ab^S (SS) lines. Neonates from Cry1Ab^R and Cry1Ab^s colonies, F_1s, and reciprocal backcrosses were exposed to a diagnostic 200.0 ng Cry1Ab toxin × cm^{-2} dose of for 7 d as described above. Dominance of relative fitness (D_{WT}) conferred by the resistance allele(s) of the Cry1Ab^R strain when subjected to Cry1Ab toxin overlays of 200 ng cm^{-2} was calculated an interpreted as a relative measure of dominance level of fitness (h) according to Bourguet et al. (2000).

2.2. Correlation of apn transcript level with Cry1Ab resistance

Midguts were dissected from twelve 5th instars from the Cry1Ab^S, Cry1Ab^R, F_1, and reciprocal backcross progeny (generated by mating *en masses* in the prior section), flash frozen in liquid nitrogen, and total RNA extracted as described by Coates et al. (2008b). RNA was quantified on a Nanodrop ND-2000 (Thermo Scientific, Wilmington, DE) at 240 nm and ~750 ng was used template for 1st strand cDNA synthesis using the Abgene Reverse-IT kit according to manufacturer instructions. Real time RT-PCR assays were run for *O. nubilalis apn*1 (*onapn*1), *onapn*2, *onapn*3, and *onapn*4 in triplicate 25 μl real-time RT-PCR reactions that included 12.5 μl iQ SYBR Green reaction mix (BioRad, Hercules, CA), 5.0 pmol each of primer (Table S1), and 2.0 μl 1st strand cDNA (diluted 1:10 with nuclease free water). Reactions were amplified on an iQ thermocycler (BioRad) at 95 °C for 3 m, then 40 cycles of 95 °C 30 s, 60 °C for 30 s, and 72 °C for 10 s followed by melt curve analysis (55 °C for 10 s, +0.5 °C/cycle for 80 cycles) with fluorescence measured at 490 nm. Cycle threshold (C_T) was estimated from background subtracted data and transcript levels were normalization to β–actin using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The relationships between transcript levels and proportion of genetic background from the Cry1Ab^R colony from Cry1Ab^S, Cry1Ab^R, F_1, and reciprocal backcross groups was assessed with the Pearson product–moment correlation coefficient (PMCC) using SAS 9.2 (SAS Institute). Data were comprised of mean transcript levels from individual samples estimated from RT-PCR reactions run in triplicate of each aminopeptidase N gene, and replicated across 4 larval samples for each of the Cry1Ab^S, Cry1Ab^R, F_1, and reciprocal backcross groups.

2.3. Mapping of Cry1Ab resistance in O. nubilalis

2.3.1. Physical mapping of the aminopeptidase N gene family

Oligonucleotide primers were designed to amplify *onapn*2 (GenBank accessions EU686652.1–EU686655.1) and *onapn*5 genes

from *O. nubilalis* (GenBank accession JF308287) using Primer3 (Table S1). The *onapn*1, *onapn*2, *onapn*3, *onapn*4 and *onapn*5 gene fragments were PCR amplified according to Coates et al. (2008b, 2009) to screen the *O. nubilalis* BAC library, OnB1, using methods described by Coates et al. (2009). DNA from OnB1 library clones were isolated and quantified as described by Coates et al. (2009), and ~0.5 μg digested with 0.5 U of *Eco*RI, *Pst*I, or *Eco*RI + *Pst*I for 5 h. Entire digested products were separated on a 0.8% TAE gel at 50 V for 8 h using Lambda *Eco*RI + *Hind*III digest as a molecular weight standard, and fragment sizes estimated using Chemidoc XRS software (BioRad). Fragment size estimates from gel images were entered into the program FPC and restriction maps assembled in default parameters (Soderlund et al., 1997).

OnB1 library clones 04G20, 50L07, and 55M15 were cultured, and DNA isolated and sequenced on a Roche 454 at the University of Illinois, William H. Keck Center for Comparative and Functional Genomics as described by Coates et al. (2012). Raw data was assembled using GS *De Novo* Assembler v2.3 default parameters (Seed step: 12, Seed length: 16, Min overlap length: 40, Min overlap identity: 90%, Alignment identity score: 2, and Alignment difference score: 23), and all of the non-redundant contigs were exported to a file in FASTA format by software provided by the manufacturer. Contig data was imported into a local nucleotide database and queried with *Bombyx mori* aminopeptidase N protein sequences from gene models BGIBMGA001641, BGIBMGA001642, BGIGMGA008017, BGIBMGA008018, and BGIBMGA008059 thru BGIBMGA008063 using the tblastn algorithm, and results filtered for hits with *E*-values $\leq 1 \times 10^{-25}$. Gene coding regions were annotated within contig sequence using the program Sequin and submitted to the GenBank nr database.

2.3.2. Mapping quantitative trait loci involved in Cry1Ab resistance

A biphastic linkage mapping approach was used as described by Heckel et al. (1999) to establish pedigrees initiated from a single parental mate pair of a Cry1Ab resistant female (rr♀; $P_{rr♀}$) × susceptible male (SS♂; $P_{SS♂}$), and subsequent backcross families that were derived from an F_1 male × Bt resistant female ($F_{1rS♂}$ × $BCP_{rr♀}$; pedigree BC3C) or a reciprocal F_1 female × resistant male ($F_{1rS♀}$ × $BCP_{rr♂}$; pedigree BC4E). A total of 28 neonates from each backcross were reared on semi-meridic diet (untreated controls). All remaining reciprocal backcross progeny were phenotyped following a 7-day sub-lethal toxin exposure as described by Coates et al. (2007). DNA was extracted from initial parents $P_{rr♀}$ and $P_{SS♂}$, BC3C and BC4E backcross parents, and untreated control and Cry1Ab-phenotyped individual using the Qiagen Blood and Tissue Extraction Kit (Qiagen, Valencia, CA) according to manufacturer instructions and quantified on a Nanodrop ND-2000 (Thermo Scientific, Wilmington, DE).

DNA was prepared for amplified fragment length polymorphism (AFLP) assays and used with 24 selective primer pairs in AFLP-PCR as described by Coates et al. (2011a). DNA not used for AFLP template generation was diluted to 10 ng μl^{-1} using deionized nuclease free water, and genotyped using SNP markers previously developed for the candidate Bt-resistance genes *brainiac* (*brn*), cadherin (*cad*), and aminopeptidase N (*apn*1 and *apn*3) (Coates et al., 2005, 2007, 2008a,b). Additionally, 763 *O. nubilalis* SNP markers were assayed Sequenom MassARRAY® at the Iowa State University Center for Plant Genomics (ISU-CPG; Ames, IA, USA) as previously described by Coates et al. (2011b). Genotypes from AFLP and SNP markers segregating among backcross progeny from heterozygous $F_{1rS♀}$ (backcross BC3C) or $F_{1rS♂}$ (backcross BC4E) and a corresponding homozygous backcross parents $BCP_{rr♂}$ (BC3C) or $BCP_{rr♀}$ (BC4E) were pooled by backcross, and predicted 1:1 Mendelian genotypic ratios inheritance were tested using the χ^2 statistic (P cutoff > 0.05). Mendelian inherited SNP markers were input into MAPMAKER 3.0 (Lincoln et al., 1992) and linkage associations among markers were determined using the Kosambi mapping function with cutoffs of LOD ≥ 2.0 and $r \leq 0.35$. Graphics created using MapDraw v. 2.1 (Liu and Meng, 2003).

3. Results

3.1. Dose-response assays and dominance of Cry1Ab resistance

Results of Cry1Ab toxin overlay assay indicated that the estimated LC_{50} for larvae $Cry1Ab^S$ (9.2 ± 1.6 ng Cry1Ab toxin × cm^{-2}) was >1359-fold lower compared to $Cry1Ab^R$ (Fig. S1). Confidence intervals for the LC_{50} estimate from the $Cry1Ab^R$ strain could not be calculated due to insufficient larvae mortality even after high Cry1Ab toxin exposures. Regardless, the lower level of the confidence interval at 12,500.0 ng Cry1Ab toxin × cm^{-2} was used to extrapolate an LC_{50} value of ≤13,853 ng Cry1Ab toxin × cm^{-2}. The LC_{50} value for the $Cry1Ab^R$ strain was significantly higher than that estimated for $Cry1Ab^S$ (Student's T-test statistic = 4.02 × 10^{-104}, P-value = 0. 0005). The LC_{99} estimate for of the $Cry1Ab^S$ strain was 97.5 ± 2.1 ng Cry1Ab toxin × cm^{-2}, and was used to set a minimum dose of 200 ng Cry1Ab toxin × cm^{-2} that as the diagnostic dose to separate resistant and susceptible phenotypes.

Exposure of neonates from the $Cry1Ab^S$ strain and backcross progeny from F_1♀ × $Cry1Ab^S$♂ crosses to a diagnostic toxin concentration of 200 ng Cry1Ab toxin × cm^{-2} resulted in zero and ≤6.25% survivorship after a 14 d exposure. In contrast, larvae from F_1, F_1 × $Cry1Ab^R$ backcross and the $Cry1Ab^R$ strain respectively showed 37.82 ± 11.39%, 77.31 ± 6.81%, and 93.75% survival (Fig. 1). The slope of the survivorship curve showed a positive correlation

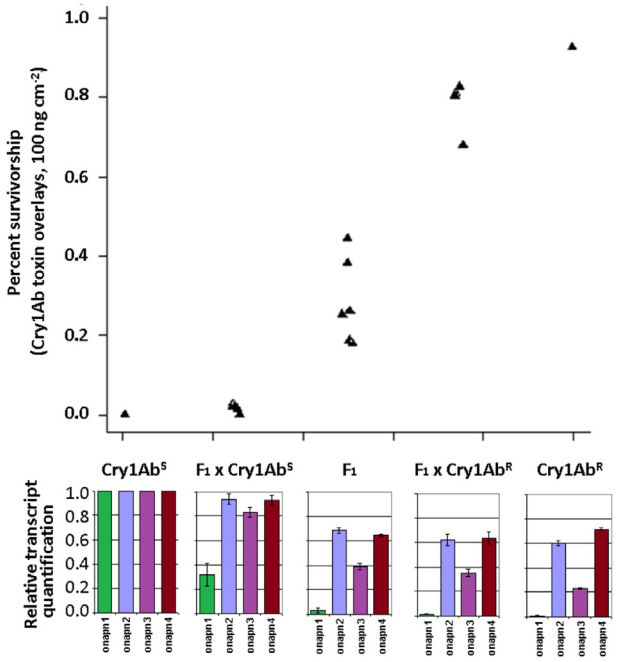

Fig. 1. Correlation between larval survival *onapn*1 transcript expression among line cross progeny and increasing proportions of Cry1Ab toxin resistant genetic background. (A) The percent survivorship phenotype among neonates from resistant ($Cry1Ab^R$), susceptible ($Cry1Ab^S$), F_1 hybrid, and reciprocal backcross lines (F_1 × $Cry1Ab^R$ and F_1 × $Cry1Ab^S$) after 14 d exposure to 100 ng Cry1Ab × cm^{-2}. (B) Relative real time RT-PCR quantification of *onapn*1 transcripts within midgut-derived cDNA from progeny of line crosses. All estimations were normalized by levels of the β-actin house keeping gene, and relative *onapn*1 transcript levels were make with respect to that within the $Cry1Ab^S$ strain using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Standard error reported among biological replications with lines.

with increasing proportions of genetic background from the Cry1Ab^R strain, and is consistent with a partially additive genetic component of the trait. Thus, if the phenotypic value of the homozygote (rr) = $2a$ = 1.0000, then the corresponding phenotypic value of the heterozygote (rS) = 0.3782 ÷ 0.9375 = 0.4034 compared to 0.5 for pure additive traits. Incomplete dominance, $D_{WT} = h = 0.40$, was demonstrated for the Cry1Ab resistance trait using survivorship percentages among the Cry1Ab^R, Cry1Ab^S and F_1 heterozygote groups.

3.2. Correlation of apn transcript level with Cry1Ab resistance

3.2.1. Estimating apn transcript level in different genetic backgrounds

Real-time RT-PCR data showed that relative *onapn*2, *onapn*3, and *onapn*4 transcript levels in midgut tissue had a ≤6-fold difference between phenotypes (Fig. 1). In contrast, *onapn*1 transcript levels were ~3-, 34-, 91-, and 147-fold lower in midgut cDNA respectively derived from F_1 × Cry1Ab^S backcross, F_1 × F_1, F_1 × Cry1Ab^R backcross, and Cry1Ab^R larvae, (estimates relative to Cry1Ab^S larvae). Specifically, *onapn*1 transcript levels were ~146.2 ± 17.4-fold higher among susceptible Cry1Ab^S compared to the resistant Cry1Ab^R strain (Table 1A). A 7.1 ± 1.9-fold reduction in midgut *onapn*3 transcript level in Cry1Ab^R compared to Cry1Ab^S was also significant (Table 1B; *T*-test statistic 9.038 $P \leq 0.001$). The *onapn*3 transcript levels were significantly reduced in F_1, F_1 × Cry1Ab^R, and Cry1Ab^R larvae midgut tissue compared to *onapn*2 or *onapn*4 (pairwise *T*-test statistic ≥ 84.4, $P \leq 0.0260$), but not *onapn*1 transcripts (*T*-test statistic = 49.0, $P = 0.0029$).

3.2.2. Association between reduced apn transcript levels and Cry1Ab resistance

The mean transcript level estimated among Cry1Ab^S, F_1, and reciprocal backcross progeny were reduced for all aminopeptidase N genes, but the decrease was most pronounced for *onapn*1 and *onapn*3 (Fig. 1). The PMCC analysis of these data showed a strong correlation between of mean *apn*1, transcript level mean survivorship among larvae from Cry1Ab^S and Cry1Ab^R colonies ($R^2 = 0.99$; $P < 0.001$), as well as among groups of larvae with increasing levels of genetic background from the Cry1Ab^R colony Fig. 1; $R^2 = 0.76$; $P < 0.001$). Similarly, *apn*3 transcript levels also showed a significant correlation with increasing proportion of Cry1Ab^R background ($R^2 = 0.91$; $P < 0.001$). No other transcript levels showed any significant correlations (remaining results not shown). Since increasing genetic background from the Cry1Ab^R colony was positively correlated with increased survival on Cry1Ab toxin bioassays, corresponding correlation between decreased *onapn*1 and *onapn*3 transcript levels and increasing genetic background from the Cry1Ab^R colony was extrapolated to also include association between transcript levels and Cry1Ab resistance.

3.3. Mapping of Cry1Ab resistance in O. nubilalis

3.3.1. Physical mapping of the aminopeptidase N gene family

Twelve BAC clones from the OnB1 library were identified using *onapn*1, *onapn*2, *onapn*3, *onapn*4 and *onapn*5 markers (37 PCR positives, mean of 2.65 ± 0.84 markers per insert; Fig. 2A). BAC fingerprints from 9 of 12 clones (75%) were used to generate an approximate 182 kb physical map representative of the *O. nubilalis* genome interval and determine a minimum tiling path for subsequent DNA sequencing (Fig. 2B). A total of 20.4 and 17.1 Mb of raw Roche GS-FLX data was obtained from libraries created for OnB1 clone 04G20 and 55M15, and was assembled into 3 contigs using the Newbler Assembler; 2 from clone 04G20 (GenBank accessions JF339038 and JF339039) and a single contig from 55M15 (JF339040). Query of BAC contigs with *onapn*1, *onapn*2, *onapn*3, *onapn*3a, *onapn*4 and *onapn*8 cDNAs using the blastn algorithm, and *B. mori* aminopeptidase N proteins using the blastx algorithm

Table 1
Relative real-time RT-PCR quantification of midgut tissue transcript levels for A) *onapn*1 and B) *onapn*3 transcripts.

Colony	Mean *onapn*1 C_T	Mean β-actin C_T	ΔC_T (Avg. *onapn*1 C_T − avg. β-actin C_T)	$\Delta\Delta C_T$ (Avg. ΔC_T − avg. $\Delta C_{T,\ Cry1AbR}$)	Normalized *onapn*1 relative to Cry1Ab^R
A) *onapn*1 transcript levels					
Cry1Ab^R	28.5	27.0			
	28.3	26.6			
	28.7	26.7			
	28.6	27.0			
	28.6	26.9			
	28.3	26.8			
Avg.	28.50 ± 0.15	26.83 ± 0.15	1.67 ± 0.30	0.00 ± 0.30	1.02 ± 0.21
Cry1Ab^S	24.4	30.0			
	24.4	30.1			
	24.5	29.9			
	24.6	30.0			
	24.3	30.0			
	24.5	29.8			
Avg.	24.45 ± 0.10	29.97 ± 0.09	−5.52 ± 0.17	−7.18 ± 0.17	146.21 ± 17.39
B) *onapn*3 transcript levels					
Cry1Ab^R	26.1	26.8			
	26.3	27.0			
	26.2	26.6			
	26.3	26.7			
	26.1	27.0			
	26.0	26.9			
Avg.	26.17 ± 0.11	26.83 ± 0.15	1.67 ± 0.30	0.00 ± 0.30	1.02 ± 0.21
Cry1Ab^S	26.9	30.0			
	26.3	30.1			
	26.0	29.9			
	26.3	30.0			
	26.8	30.0			
	26.8	29.8			
Avg.	26.52 ± 0.33	29.97 ± 0.09	−3.45 ± 0.39	−2.78 ± 0.39	7.10 ± 1.90

Cry1Ab^R and Cry1Ab^S are respectively the *B. thuringiensis* Cry1Ab toxin resistant and susceptible lines.

	Bacterial artificial chromosome (BAC) clones from the *Ostrinia nubilalis* library, OnB1											
Gene	04G20	50K02	50L07	52G07	53A02	55D13	55M15	55O13	67O24	72G03	74F22	74N03
*onapn*1	+	+			+	+				+	+	
*onapn*2							+		+			
*onapn*3	+	+	+	+	+	+		+	+	+	+	+
*onapn*4	+	+		+	+	+	+	+	+	+	+	+
*onapn*5			+				+		+			

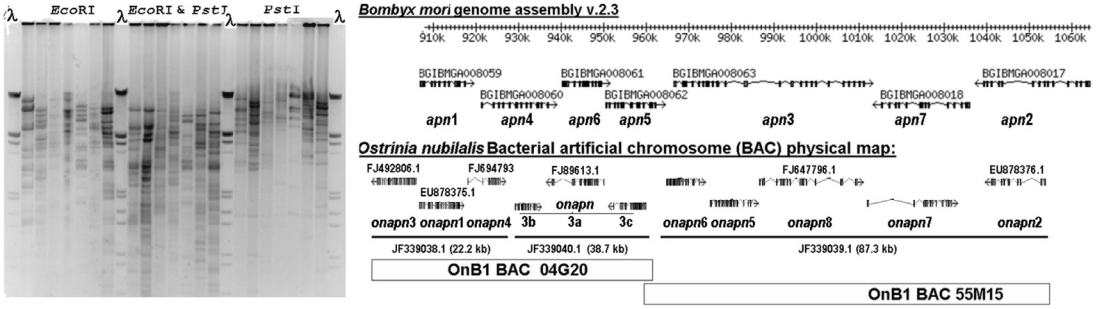

Fig. 2. Physical map of the *Ostrinia nubilalis* genome interval encoding the aminopeptidase N gene family that has resulted from a series of tandem duplications. (A) Identification of 12 bacterial artificial chromosome (BAC) clones from the *O. nubilalis* library OnB1 that contain genome regions with ≥2 gene intervals for *onapn*1, *onapn*2, *onapn*3, *onapn*4 or *onapn*5. (B) Representative agarose gel used to fingerprint BAC inserts by *Eco*RI, *Pst*I, and *Eco*RI and *Pst*I double restriction endonuclease digestion. Lambda (λ) *Eco*RI and *Hind*III double restriction endonuclease digestion product was used for size comparison. (C) Comparative alignment of the 0.88–1.65 Mb interval of the *Bombyx mori* chromosome 9 that contains seven unique aminopeptidase N gene models with the physical map of OnB1 BAC inserts from clones 04G20, 50L07, and 55M15. The position of contigs assembled from full insert sequences of OnB1 clones 04G20 and 55M15, along with intron-exon structure of full gene intervals for *onapn*1, *onapn*2, *onapn*3, *onapn*3a, *onapn*4, *onapn*5, *onapn*6, *onapn*7 and *onapn*8 are shown.

resulted in the prediction of intron-exon structure for *onapn*3b, *onapn*3c, *onapn*5, *onapn*6, and *onapn*7 genes (Fig. 2C).

3.3.2. *Quantitative trait loci involved in Cry1Ab resistance*

Inheritance of larval *O. nubilalis* Cry1Ab resistance was evaluated within a biphasic pedigree, which exploits the a lack of meiotic crossover during lepidopteran oogeneisis to identify resistant female-derived markers that co-segregate with the trait. Pedigrees BC3C chromosomes were inherited as a single haplotype from the initial resistant female parent ($P_{rr♀}$) through all of backcross progeny. A total of 156 SNP markers were heterozygous in the $F_{1♀}$ parent and homozygous $BCP_{♂}$ resistant line parent, and thus segregating among BC3C backcross progeny. Chi-square (χ^2) tests indicated that genotypes from 140 markers (File S1) did not significantly deviated from a predicted 1:1 Mendelian proportion among 22 backcross progeny reared on control diet ($P \geq 0.0887$; Table S2). Genetic linkage analysis of Mendelian-inherited SNP markers from control group progeny resulted in a map consisting of 126 markers on 31 linkage groups (LGs; 4.06 ± 2.24 SNP markers per LG; Table S3).

Analogous genotypes from full-sib BC3C progeny that had molted to 2nd instar on Cry1Ab diet (Cry1Ab resistant group; File S1) had 11 makers of these 140 SNP markers which were non-Mendelian. Seven of the 11 non-Mendelian SNP markers (63.6%) were on LG24 ($P \leq 0.01058$) and 3 (27.3%) were on LG27 ($P \leq 0.0493$; χ^2-statistic results in Table S2). One non-Mendelian marker, Contig05855.192, showed no linkage to any other marker. A plot of the mean deviation in genotypic proportions for SNP markers located on the 31 *O. nubilalis* LGs showed that markers on LG24 significantly deviated from Mendelian expectation ($P_{mean} = 0.0059 \pm 0.0023$) as did markers on LG27 ($P_{mean} = 0.0403 \pm 0.0160$; Fig. 3). The significance of deviations from Mendelian expectations did not surpass a Bonferroni adjusted α ($\leq 0.05/31 = 0.0016$) for either LG24 or LG27. Results also showed that SNP in candidate Bt toxin binding protein genes segregated independent of LG24 or LG27, and showed no significant deviation from Mendelian proportions among Cry1Ab survivors. Specifically, *onapn*1 and *onapn*4 mapped to LG02, brainiac/bre5 to LG03, ABC transporter to LG12, and cadherin to LG14, and alleles from the initial resistant female were equally likely among backcross progeny from Cry1Ab or control diet (Table S3).

Pedigree BC4E consisted of an $F_{1♂}$ parent backcrossed to a resistant $BCP_{♀}$ parent, and the resulting BC4E progeny were recombinants of the Cry1Ab resistant haplotypes inherited from the initial Cry1Ab resistant female parent ($P_{rr♀}$). SNP genotyping of BC4E backcross parents and progeny was conducted only for multiplex reactions that contained markers linked to the QTL for Cry1Ab (LG24), and a set of 24 AFLP primer pairs that were used to increase marker density on LG24. Analysis of SNP and AFLP genotypes from BC4E untreated control progeny (File S2) showed that SNP markers Contig00519.709, Contig03610.309, Contig05998.290, and Contig06606.695 along with 2 AFLP markers E-ACC700_M-TG0160 and E-AGC800_M-TG0177 were Mendelian (*P*-values ≥ 0.1573; Table S4) and co-segregating on LG24 (22.46 cM genome interval; 4.5 ± 4.72 cM between markers; Fig. 4). Data from the same markers from BC4E larvae that had molted to 2nd instar on Cry1Ab toxin diet (File S2) showed that Contig03610.309, Contig05998.290, E-ACC700_M-TG0160, and E-AGC800_M-TG0177, deviated significantly from expected 1:1 Mendelian ratios (*P*-values ≤ 0.0310; Table S4), with the greatest deviation estimated for AFLP marker E-ACC700_M-TG0160 ($P = 0.0039$) that surpassed the Bonferroni adjusted $\alpha = 0.0016$ (Fig. 4). In contrast, no significant deviation was detected for markers on LG27 in backcross BC4E, Contig00519.709 ($P = 0.0599$) or Contig06606.695 ($P = 0.1823$), despite an indication of significance in backcross BC3C.

4. Discussion

Transgenic crops that express Bt crystalline insecticidal protein toxins have shown remarkable success at suppressing pest insect damage in instances where “high-dose” events are capable of causing mortality among 100% of homozygous susceptible and heterozygous individuals (Huang et al., 2011). In contrast, resistance rapidly developed to “low-dose” events within populations of the coleopteran insect, *Diabrotica virgifera virgifera* (Gassmann et al., 2011). Success of IRM strategies for Bt crops has also benefitted from resistant insect phenotypes that have an

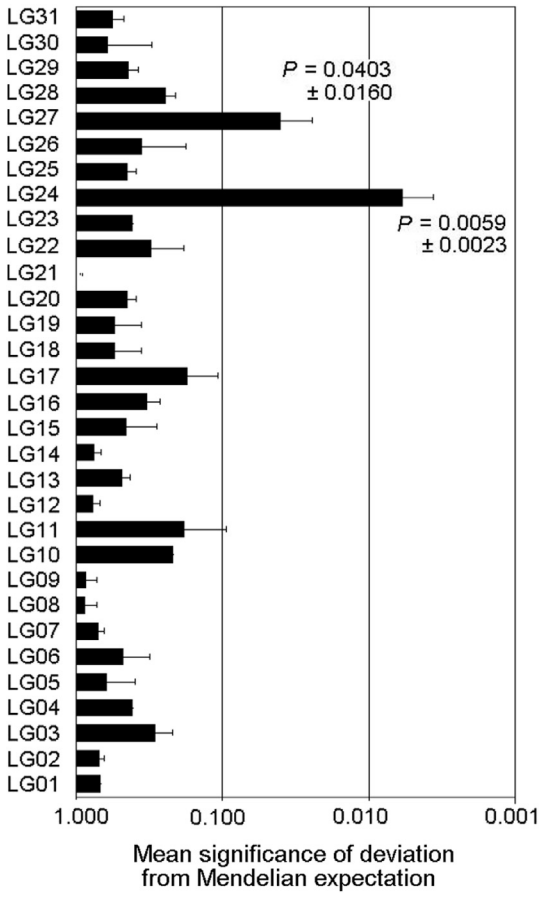

Fig. 3. Quantitative trait locus (QTL) mapping that show the influence of linkage groups 24 (LG24) and 27 (LG) on inheritance of an *Ostrinia nubilalis* Cry1Ab resistance trait. In spite of co-segregation of an ~146-fold reduction in midgut aminopeptidase N 1 (*apn*1) transcript levels with Cry1Ab resistance, the single major QTL for the trait is independent of markers for tandem duplicated *apn* gene family members (*onapn*1, *onapn*3, and *onapn*4) on LG02. The candidate resistance genes cadherin and ABC transporter constitute putative Bt binding receptors and are mapped to LG12 and LG14, respectively.

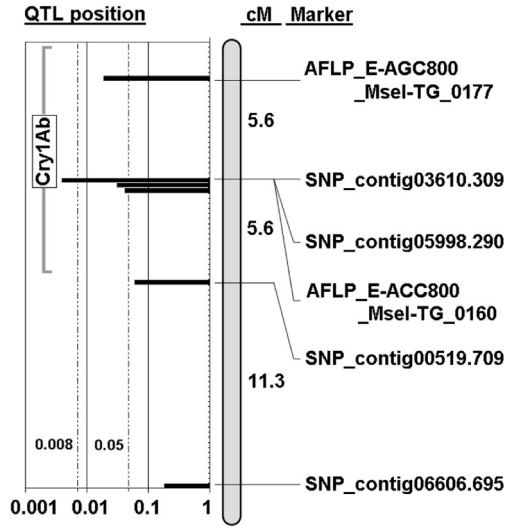

Fig. 4. Positional mapping of the major quantitative trait locus (QTL) determining Cry1Ab resistance in *Ostrinia nubilalis* larvae. An ~12 cM interval on linkage group 24 (LG24) from pedigree BC4E contains four segregating markers that are significantly skewed from Mendelian expectation ($\alpha \leq 0.05$) and a single marker, SNP_contig03610.309, that crossed a Bonferroni adjusted significance threshold ($\alpha = 0.05/6 = 0.008$).

effective dominance (h) of zero, wherein heterozygote genotypes are functionally susceptible. In recent years, lepidopteran insect populations have evolved resistance traits that enable larval survival and development on transgenic Bt crops in field conditions (Kranthi, 2005; van Rensburg, 2007; Li et al., 2007; Matten et al., 2008; Tabashnik et al., 2008), where dominant alleles have been characterized among Cry1Ac cotton resistant *Helicoverpa armigera* and *Helicoverpa zea* (Tabashnik et al., 2008; Zhang et al., 2012). A similar incomplete recessive nature of Bt resistance was also described among *Plutella xylostella* that survived exposure to foliar Bt sprays (Tabashnik et al., 1997), as well as among laboratory selected Cry1C resistant *Spodoptera littoralis* (Chaufaux et al., 1997) and Cry1Fa resistant *O. nubilalis* (Pereira et al., 2008). Although estimates of relative fitness depends upon conditions in which the trait is measured, the Cry1Ab toxin resistance trait in the *O. nubilalis* Cry1Ab^R colony ($D_{WT} = h = 0.40$) suggests that resistant alleles are nearly co-dominant. The observation of consistent field suppression of *O. nubilalis* populations (Hutchison et al., 2010) suggests that current commercialized "high-dose" Cry1Ab events may cause this trait to be irrelevant in terms of resistance evolution (Bourguet et al., 2000), but provide an additional instances where complete recessive behavior of resistance alleles cannot be assumed under the HDR strategy.

The genetic basis of Cry1A and Cry1F resistance traits have been shown to be controlled by single genes or genetic loci (Gahan et al., 2001, 2010; Morin et al., 2003; Baxter et al., 2008; Coates et al., 2011a,b; Zhang et al., 2012). In contrast, Cry1Ac resistance in the laboratory-selected *H. virescens* YHD2 strain was shown to be polygenic through the involvement of *cad* and *abcc*2 genes (Gahan et al., 2001, 2010). Chronic exposure to low levels of Bt toxin in laboratory selection schemes have been suggested to favor the accumulation of insecticide resistance traits based upon many genes of small effect (polygenic; Groeters and Tabashnik, 2000). Similarly, the *O. nubilalis* Cry1Ab^R stain was exposed to sub-lethal levels of Cry1Ab toxin during selection (Coates et al., 2007), such that the additive genetic component predicted to involve >1 gene could be an artifact of laboratory selection. Regardless, the *O. nubilalis* Cry1Ab resistance described in this research may provide a model for the genetic effects of sub-lethal exposure on target pest insects, any might have relevant analogies to field selection from "low-dose" transgenic corn events that are currently used (e.g. Bt corn hybrids commercialized for the control of *D. v. virgifera*).

The molecular basis of Bt resistance traits in Lepidoptera have been linked to mutations that cause changes in protein structure or transcript abundance (and corresponding protein abundance) compared to susceptible individuals (see Introduction). A constitutive decrease in *apn*1 transcript level was correlated with the Cry1Ab toxin resistance trait in the *O. nubilalis* Cry1Ab^R strain in our experimental design, and was also previously shown to be the basis of Cry1Ca resistance in *S. exigua*, and Cry1Ac resistance in *H. armigera* (Zhang et al., 2009) and *T. ni* (Tiewsiri and Wang, 2011). Similar to *onapn*1, our results showed that *onapn*3 transcripts decreased in response to increasing Cry1Ab^R genetic background and were also likewise significantly correlated with inheritance of Cry1Ab resistance trait. The fold reduction and degree of *onapn*3 linkage to Cry1Ab resistance was lower compared to *onapn*1, which could suggest that *onapn*1 and *onapn*3 might share common regulatory control factors. Furthermore, the potential intertwined regulatory control as well as tight genetic linkage between *onapn*1 and *onapn*3 (see below) may interject difficulties in the dissection of independent contributions of these genes to the Cry1Ab^R

resistance trait. These reports suggest that mutations which affect the transcription of membrane-bound aminopeptidase N genes is likely to decrease subsequent translation such that the protein receptor is effectively eliminated it from the midgut epithelium. The reoccurrence of *apn*1 transcript suppression as a potential mechanism for Cry1 toxin resistance suggests that APN1 (and potentially APN3) is a critical binding receptor in the Cry1 toxin mode of action in Lepidoptera. Indeed, both *apn*1 and *apn*3 orthologs are known to bind Cry1A toxin in other species of Lepidoptera (reviewed by Piggot and Ellar, 2007). Post-translational glycosylation of *T. ni apn*3 was shown to be a requirement for Cry1Ac binding (Gill et al., 1995; Garner et al., 1999), and suggested that *in vivo* insect cell expression systems are necessary for proper binding analyses. Subsequent expression of *H. armigera apn*3 in *T. ni* cell lines demonstrated the capacity to bind Cry1Ac, but not Cry1Ab or Cry1Aa (Rajagopal et al., 2003). Similar results were obtained from purified endogenous *apn*3 from *L. dispar* (Valaitis et al., 1997). In contrast, the *O. nubilalis apn*3a isoform was shown only to bind Cry1Fa when expressed in Sf21 cells (Crava et al., 2013). *apn*1 has repeatedly been shown to be involved in Cry1A resistance mechanisms of lepidopteran species (see Introduction). Since the control of gene expression can be enacted through a network of interconnected genetic factors or multiple pathways that are often co-regulated within a biological system (Jovelin and Phillips, 2009), genes within the *apn* gene family may retain shared ancestral regulatory mechanisms. It is conceivable that correlation of decreased *onapn*3 transcription with Cry1Ab resistance may be a consequence of placement within the same gene regulatory network as *onapn*1, and that correlation of *onapn*3 with Cry1Ab resistance results from mutations in *trans*-acting regulatory factors that modulate the major Cry1Ab binding receptor (*onapn*1). Understanding the genetic control of *apn* transcripts may be important for deciphering of mechanism(s) involved in Cry1 resistance in lepidopteran species, and will be a focus of future studies.

Using evidence from the segregation of genetic markers, Coates et al. (2008a) previously showed that the *onapn*1 locus was independent of the Cry1Ab resistance trait in the same *O. nubilalis* Cry1Ab^{R} strain used in this study. This was not surprising since loci for candidate Bt binding receptors have been shown not to be linked to associated resistance traits (Baxter et al., 2008; Coates et al., 2011a,b), but such studies have the major drawback of limiting the genomic loci at which associations can be made with the trait (Tabor et al., 2002; Wayne and McIntyre, 2002). In our current analyses the genetic location of *onapn*1 was predicted on LG2, which was unlinked and segregating independently of QTL identified on LG24 and LG27. These QTL results confirmed previous observations by Coates et al. (2008a), as well as our phenotypic evidence which suggested that Cry1Ab resistance in the Cry1Ab^{R} colony has an additive (polygenic) genetic component. QTL results also suggested that genetic factors on LG24 and LG27 may be controlling transcription of *onapn*1 on LG2. Specifically, mutations that decrease the transcription at a genetic locus may be caused by changes in *cis*- or *trans*-regulatory mechanisms. Since genes are physically linked to proximal *cis*-regulatory elements (promoters or enhancers) chromosomal segregation or recombination are not likely to cause significant linkage disequilibrium between the causal gene and QTL. In contrast, *trans*-regulatory control of a gene can be mediated by soluble protein factors that bind *cis*-promoter or enhancer elements, such that gene products that act as *trans*-regulatory factors (transcription factors) move within the nucleus to affect transcription at unlinked loci. Although not a novel concept in genetics, the control of transcription for a Bt toxin receptor and associated resistance traits by putative mutations in *trans*-acting regulation has only been described once previously (Tiewsiri and Wang, 2011), but reinforces that complexities of gene interactions (epistasis) can contribute to phenotypic variance.

Since the reduced expression of *apn*1 has a role in Cry1 resistance traits from multiple lepidopteran species, deciphering the genetic control of this gene in Lepidoptera may be important in order to understand the evolution of these resistance mechanisms. Seven aminopeptidase N genes are duplicated in tandem in the *B. mori* genome, which agreed with prior inference that a duplication of *apn* genes was ancestral to the lepidopteran lineage (Chang et al., 1999; Angelucci et al., 2008). Seven *apn*–like paralogs were defined from *O. nubilalis* cDNA (Crava et al., 2010) and were subsequently identified in physically overlapping genomic inserts from two *O. nubilalis* BAC clones in this study. When compared to *B. mori* genome assembly, a lineage-specific duplication of *onapn*3 was suggested by prediction of the *onapn*3 gene (Crava et al., 2010) and confirmed by our BAC insert sequences that contained *onapn*3a and *onapn*3b, but assembly predicted presence of a third paralog, *onapn*3c. This suggest that sequencing of genome intervals might be more successful for the identification of genes compared to cDNA-based methods due to lack of transcriptional dependence.

5. Conclusions

Mutations that create variance in gene interactions (epistasis) in a population lead to novel phenotypes that are manifested within the context of a given environmental condition, therefore differences in transcription-level regulation and interactions among different alleles in unique genome architectures contribute to phenotypic diversity (West et al., 2007). Regulatory mutations have previously been shown to be the likely cause of a Cry1Ac resistance trait (Tiewsiri and Wang, 2011), but in the currently study we uniquely show that two independent genetic loci affect the co-regulation of *apn*1 and *apn*3 transcription occurs in *trans*. Furthermore, control of expression in the aminopeptidase N gene network shows interconnectivity through the co-regulation of *onapn*1 and *onapn*3. Unraveling the mechanism used to regulate the expression of this gene family may lead to a better understanding of the genetic basis of Cry1A resistance in Lepidoptera.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.09.003.

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